Seed health and germination of *Brassica* spp. from seed companies in South Africa

G. Mandiriza[1,2], Q. Kritzinger[1] and T. Aveling[1,2, a]
[1]Department of Plant and Soil Sciences, University of Pretoria, Pretoria, South Africa; [2]Forestry and Agricultural Biotechnology Institute, Pretoria, South Africa[b].

Abstract

Seed-borne pathogens found on brassica seeds can cause significant reduction in crop productivity and quality. In this study, seed health tests and germination of brassica seeds collected from several seed companies in South Africa including a farm retained sample were evaluated. Fungi detected from 13 seed samples using the agar plate method included *Alternaria brassicicola, A. alternata, Aspergillus niger, A. glaucus* and *Penicillium* spp. *A. alternata*, the most common fungal species isolated, was found in seven samples with an incidence ranging from 0.5-6.25%. Results of the pathogenicity tests of the *Alternaria* species showed that only the *A. brassicicola* isolate produced necrotic lesions on rape and cabbage seedling leaves. No seed-borne bacteria reported to be pathogenic to brassicas were detected from the seeds. Germination of seeds was evaluated according to the International Seed Testing Association rules. Highest seed germination was observed in a cauliflower seed sample at 97% and a rape seed sample recorded the lowest at 17%.

Keywords: *Alternaria* spp, brassicas, germination, seed-borne

INTRODUCTION

The genus *Brassica* constitutes an important group of vegetables and some of the species are commonly cultivated in almost all of the provinces in South Africa. In 2013 about 132 600 tonnes of cabbage and other brassicas were produced on an area of 2 314 hectares (FAOSTAT, 2016).

The production of brassica vegetables is affected by several biotic factors with diseases also playing a significant part. Seed-borne pathogens can cause seed damage, seed rot, seed necrosis, poor germination, seedling damping-off and diseases to the seedlings and growing plants (Ismail et al., 2012; Sangvikar and Wadje, 2012). Furthermore, some seed-borne pathogens have also been reported to contaminate the soil and may continue to exist in the soil providing a continuous source of inoculum and diseases (Hasan et al., 2005). Seed-borne pathogens such as *Xanthomonas campestris* pv. *campestris* Pam. (Dow.) (*Xcc*), *Leptosphaeria maculans* (Sowerby) P. Karst), *Alternaria brassicicola* (Schw.) Wilt. and *Alternaria brassicae* (Berk.) Sacc. have been reported to be associated with brassica seeds (Chitarra et al., 2002; Kubota et al., 2006; Fernando et al., 2016).

Seed is a vital input in the production of many crops (Sangvikar and Wadje, 2012) including brassicas hence the use of pathogen-free seed is important for the control of seed-borne diseases and improved crop productivity. Seed health testing is required to be able to detect seed-borne mycoflora associated with seeds as they can be primary sources of infection in the field. Beside contamination or infection by seed-borne pathogens, assessing the germination potential of seeds is also important. This study was conducted to evaluate

[a] E-mail: Terry.Aveling@fabi.up.ac.za

the seed health and germination of brassica seeds from commercial seed companies and one farm retained sample in South Africa.

MATERIALS AND METHODS

Seed health tests

1. Source of seeds.

Thirteen untreated seed samples comprising two broccoli seed lots (*Brassica oleracea* L. var*. italica*), four cabbage seed lots (*Brassica oleracea* L. var. *capitata*), three cauliflower seed lots (*Brassica oleracea* L. var*. botrytis*), one mustard seed lot (*Brassica juncea* (L.) Czern) and three rape seed lots (*Brassica napus* L.) were obtained from various seed companies and one smallholder farmer in the Gauteng Province of South Africa.

2. Detection and identification of fungi from seeds.

All the seed samples were evaluated for the presence of seed-borne fungi using the agar plate method according to the International Seed Testing Association (ISTA) (ISTA, 2014a). After surface disinfection with 1% sodium hypochlorite (NaOCl) for three minutes and rinsing three times with sterile distilled water, the seeds were individually plated on potato dextrose agar (PDA) in Petri dishes. Petri dishes were incubated at 25°C under a 12 hour cycle of near ultraviolet light and 12 hour dark conditions. The frequency of infection of the evaluated seed samples was determined as a percentage. Fungal colonies were sub-cultured on PDA, potato carrot agar (PCA) or carnation leaf agar (CLA) and incubated as above. The different fungi were identified and identifications were confirmed by the Biosystematics Division, Plant Protection Research Institute, Agriculture Research Council, Pretoria, South Africa.

3. Detection and identification of bacteria from seeds.

Detection of *Xanthomonas campestris* pv. *campestris* (Xcc) was done using a modified ISTA method on detection of *Xcc* on *Brassica* spp. (ISTA, 2014b). Five hundred seeds from each seed sample were used instead of 10 000 due to small seed samples. A tenfold dilution series from each seed extract was done up to 10^{-5}. From each dilution 100 µL was pipetted onto the centre of a Petri dish containing a selective media for *Xcc*, mCS20ABN, in triplicate and plates were later incubated at 30°C for 4 days. To isolate other bacteria associated with the seeds a method by Cottyn *et al.* (2001) was used with slight modifications. Seeds from each seed sample were partly crushed and soaked in sterile 0.1% buffered peptone water plus 0.025% Tween 20. Tenfold serial dilutions were done as above and 100 µL from each dilution was plated onto Petri dishes containing nutrient agar (NA). Petri dishes were incubated at 25°C for 2 days. After incubation Petri dishes were examined for seed-borne bacteria and the colony forming units (cfu mL^{-1}) for each seed batch was calculated.

Distinct colonies were further purified on NA and samples were identified using Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). For each bacterial sample, the raw spectra were automatically analysed using the MALDI Biotyper 3.0 software (Bruker Daltonics, Bremen, Germany) encompassing a reference library of microbial species (Ferreira *et al*. 2010; Wieser *et al*. 2012).

Pathogenicity tests of isolates from seed

Seeds of cabbage (Drumhead variety) and rape (English Giant variety) were surface sterilized and two seeds of each were sown in a plastic pot (12 cm diameter) filled with pasteurised loamy sand soil. There were three replicate pots containing the two seeds for each fungal pathogenicity test. Seedlings were allowed to grow in the greenhouse (University of Pretoria Experimental Farm) at ± 25°C with a photoperiod of 16 hours and relative humidity of 80-85%. Pathogenicity tests were conducted on the brassica seedlings at 3-4 leaf stage Seedlings were inoculated by spraying spore suspensions adjusted to 5 x 10^6 spores mL^{-1} or sterile distilled water (control) onto leaves until runoff. As the Alternaria spp. isolated from the seeds included *Alternaria alternata* (Fr.) Keissl. and *A. brassicicola*, a

disease severity scale according to Sangeetha and Siddaramaiah (2007) for Alternaria leaf spot was used.

Standard seed germination test

The standard seed germination test was conducted according to ISTA rules (ISTA 2014a) but with slight modifications. The top of the paper method with sterile filter paper (Whatman No.1) was used. Two hundred seeds were randomly selected from each seed sample and plated in four replicates of 50 seeds. Seeds were incubated at ±22°C. At the end of incubation, the number of germinated seeds, number of normal seedlings, abnormal seedlings (deformed and diseased) and non-germinated seeds was recorded.

RESULTS

Seed health tests

The predominantly isolated fungi belonged to the genera *Alternaria, Aspergillus* and *Penicillium*. The highest fungal incidence of 9.25% was recorded on rape seed Sample K. The incidence of *A. alternata* was highest on cabbage seed Sample D at 6.25%. *Alternaria brassicicola* was associated with cabbage seed Samples D and F with incidences of 0.75% and 0.25%, respectively (Table 1).

Table 1. Incidence of seed-borne fungi on 13 brassica seed samples collected within South Africa.

		Seed-borne fungi incidence (%)					
Seed	**Sample**	**Aa**	**Ab**	**An**	**Agl**	**Pen**	**Total**
Broccoli	A	0.50	0.00	0.75	0.00	0.00	1.25
Broccoli	B	0.00	0.00	1.25	0.00	0.00	1.25
Cabbage	C	0.75	0.00	0.00	0.00	0.00	0.75
Cabbage	D	6.25	0.75	0.00	0.00	0.00	7.00
Cabbage	E	2.25	0.00	0.00	0.00	0.00	2.25
Cabbage	F	4.00	0.25	0.00	0.00	0.00	4.25
Cauliflower	G	2.50	0.00	4.25	0.00	0.00	6.75
Cauliflower	H	1.50	0.00	0.00	0.00	0.00	1.50
Cauliflower	I	0.00	0.00	0.50	0.00	0.00	0.50
Mustard	J	0.00	0.00	0.00	0.00	7.75	7.75
Rape	K	0.00	0.00	1.50	7.50	0.25	9.25
Rape	L	0.00	0.00	3.00	0.50	0.00	3.50
Rape	M	0.00	0.00	0.75	1.25	0.00	2.00

[1]Aa = *A. alternata*; Ab = *A. brassicicola*; An = *A. niger*; Agl = *A. glaucus*; Pen = *Penicillium* spp.

The maximum bacterial population of 4.2 log cfu mL^{-1} was recorded on cabbage Sample D and it was significantly higher than other seed batches. This was followed by cabbage Sample F, which had a bacterial count of 3.7 log cfu mL^{-1} (Figure 1).

No seed-borne pathogens among the bacterial isolates identified to a species level by MALDI-TOF MS, such as *Xcc* could be detected. The isolated bacteria included *Bacillus* spp., *Pantoea* spp., *Paenibacillu*s spp. and *Pseudomonas* spp.

Pathogenicity tests

None of the fungal isolates of *A. alternata* formed lesions on the leaves of cabbage and rape plants. Only *A. brassicicola* was pathogenic to cabbage and rape with severity at 52.5% and 42.5%, respectively. The symptoms appeared as dark brown necrotic spots. *A. brassicicola* was re-isolated from the diseased leaves on the inoculated plants thereby fulfilling Koch's postulates. The control plants remained healthy after inoculations.

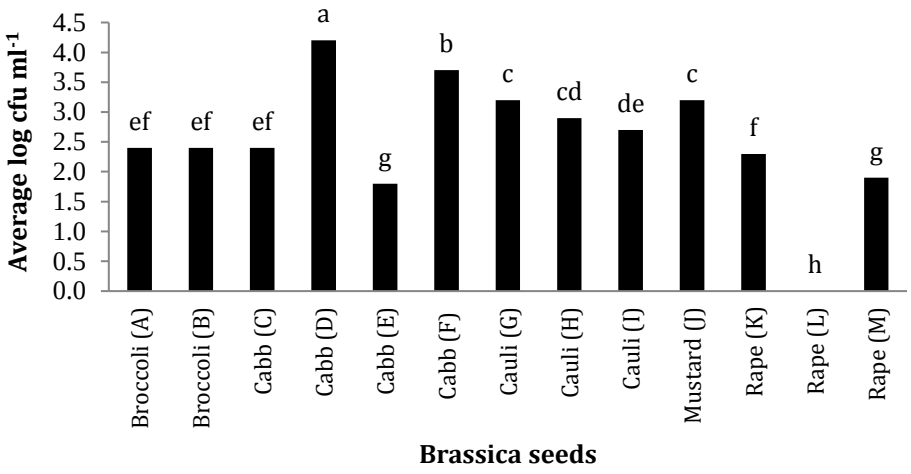

Figure 1. Bacterial populations on brassica seeds. Means with the same letters are not significantly different according to Fisher's LSD (p = 0.05). Significance: p<0.05. Cauli = cauliflower, Cabb = cabbage, (A), (B) etc. = seed sample code.

Standard seed germination tests

High seed germination percentages which were not different statistically were recorded by most of the seed samples except for broccoli seed Sample B and rape seed Sample L (stored for 2 years before the test) which recorded low germination of 68% and 17%, respectively. A significantly (p<0.05) low percentage of normal seedlings at 12% was noticeable with rape seed Sample L. Broccoli seed Sample B recorded the highest percentage of abnormal seedlings (13%). The percentage of diseased seedlings ranged from 0% to 5% (Table 2).

Table 2. Germination of brassica seed samples collected in South Africa.

Seed	Sample	*Germination %	*Normal %	*Abnormal %	*Diseased %
Broccoli	A	96.0 a	96.0 a	0.0 b	0.0 b
Broccoli	B	68.0 d	51.0 e	13.0 a	4.0 ab
Cabbage	C	76.0 cd	72.0 d	4.0 b	0.0 b
Cabbage	D	96.0 a	91.0 ab	1.0 b	4.0 ab
Cabbage	E	90.0 ab	90.0 ab	0.0 b	0.0 b
Cabbage	F	95.5 a	94.0 ab	1.5 b	0.0 b
Cauliflower	G	83.0 bc	77.0 cd	4.0 b	2.0 ab
Cauliflower	H	97.0 a	95.0 ab	2.0 b	0.0 b
Cauliflower	I	97.0 a	91.0 ab	2.0 b	4.0 ab
Mustard	J	89.0 ab	84.0 bc	4.0 b	0.0 b
Rape	K	94.0 a	88.5 ab	0.5 b	5.0 a
Rape	L	17.0 e	12.0 f	4.0 b	1.0 ab
Rape	M	92.0 ab	90.5 ab	1.0 b	0.5 b

[1]Values followed by the same letters do not differ significantly according to Fisher's LSD test (p = 0.05). [2] * indicates significance at p<0.05

DISCUSSION

In this study, *A. alternata* was the most common fungus detected from the brassica seeds. It was recovered from cabbage, cauliflower and broccoli seeds. Several studies have found *A. alternata* to be associated with brassica seeds, usually isolated from *B. oleracea* seeds (Sivapalan and Browning, 1992; Kubota et al., 2006). The fungus may exist as a saprophyte and sometimes as an opportunistic pathogen of various crops and seeds. A major seed-borne pathogen, *A. brassicicola* was detected in two cabbage seed samples at low incidences. The low incidence of *A. brassicicola* on seeds concurs with other studies where less than 10% contamination by *A. brassicicola* on seeds was reported (Tohyama and Tsuda, 1995; Kubota et al., 2006). Despite the low incidence of the fungus on seeds, contamination of seed by the pathogen can cause low seed germination, reduce seedling vigour and damping-off disease (Agarwal et al., 2004). Pathogenicity tests showed that *A. brassiciola* produced necrotic lesions on cabbage and rape plants regardless of the host, concurring with Tohyama and Tsuda (1995) who reported that the *A. brassicicola* isolates tested caused necrotic lesions on all the brassica crops tested and were not host specific.

Other fungi were also isolated from the seeds and these included *Aspergillus niger*, *A. glaucus* and *Penicillium* spp. Contamination of seeds by *Aspergillus* and *Penicillium* spp. may reduce seed quality, germination and seedling emergence (Ismail et al., 2012; Uma and Wesely, 2013). No pathogenic seed-borne bacteria such as *Xcc* were isolated from seeds but bacteria such as *Pseudomonas*, *Bacillus*, *Pantoea* and *Paenibacillus* spp. were detected. Some of these are beneficial microorganisms with biological control potential. The presence of antagonistic or saprophytic bacteria on brassica seeds have been found to lower the detection of seed-borne pathogenic bacteria on seeds (Rhandhawa and Schaad, 1984).

The germination potential of the 13 seed samples was assessed. According to Vermont Seed Standard Regulations (2014) for most brassica seeds the acceptable germination percentage is 75 %. Most seed samples had a germination percentage of greater than 80 %. This and the low incidence of fungi recorded indicated that the majority of the seed samples had good seed quality.

CONCLUSIONS

The present study reported the microflora associated with brassica seeds in South Africa. *Alternaria brassicicola* at low fungi incidences was the only seed-borne pathogen isolated from the seeds. Despite the low incidence of the pathogen and absence of other seed-borne pathogens it is important to continuously monitor seed samples from seed lots by conducting seed health and germination tests in order to ensure the quality of seeds. Such tests help reduce the introduction of pathogens into the fields, improve crop establishment and yield.

ACKNOWLEDGEMENTS

The study was funded by the European Union – TESTA Project number FP7-KBBE-2012-6-311875. Thank you to the seed companies for providing brassica seeds and to National Research Foundation (NRF) of South Africa for funding of the MALDI-TOF biotyper. Dr. M. Truter (Biosystematics Division, Plant Protection Research Institute, Agriculture Research Council, Pretoria, South Africa) is acknowledged for confirmation of the identity of the fungal isolates.

Literature cited

Agarwal, P.C., Singh, B., Dev, U., Rani, I., Rai, D., Joshi, K.D., and, D., Maurya, A.K., and Khetarpal, R.K. (2004). Interception of seed-borne pathogens in introduced planting material. Indian J. Microbiol. *44*, 37-42.

Chitarra, L.G., Langerak, C.J., Bergervoet, J.H.W., and van den Bulk, R.W. (2002). Detection of the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestri*s in seed extracts of *Brassica* sp. applying fluorescent antibodies and flow cytometry. Cytometry *47*, 118-126.

Cottyn, B., Regalado, E., Lanoot, B., De Cleene, M., Mew, T. W., and Swings, J. (2001). Bacterial populations associated with rice seed in the tropical environment. Phytopathology *91*, 282-292 http://dx.doi.org/10.1094/PHYTO.2001.91.3.282.

FAOSTAT. (2016). www. faostat.fao.org. Accessed 5/8/15.

Fernando, W.G.D., Zhang, X., and Amarasinghe, C.C. (2016). Detection of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* causing blackleg disease in canola from Canadian canola seed lots and dockage. Plants *5* (*12*), 1-11 http://dx.doi.org/10.3390/plants5010012.

Ferreira, L., Vega Castano, S., Sanchez-Juanes, F., Gonzalez-Cabrero, S., Menegotto, F., Orduna-Domingo, A., Gonzalez-Buitrago, J.M., and Munoz-Bellido, J.L. (2010). Identification of *Brucella* by MALDI-TOF Mass Spectrometry. Fast and reliable identification from agar plates and blood cultures. PLoS one *5* (*12*), 1-8 http://dx.doi.org/10.1371/journal.pone.0014235.

Hasan, M.M., Chowdhury, S.P., Shahidul, A., Hossain, B., and Alam, M.S. (2005). Antifungal effects of plant extracts on seed-borne fungi of wheat seed regarding seed germination. Seedling health and vigour index. Pak. J. Biol. Sci. *8*, 1284-1289.

Ismail, M., Anwar, S.A., ul-Haque, M.I., Iqbal, A., Ahmad, N. & Arain, M.A. (2012). Seed-borne fungi associated with cauliflower seeds and their role in seed germination. Pak. J. Phytopathol. *24* (*1*), 26-31.

International Seed Testing Association (ISTA). (2014a). Proceedings of the International Seed Testing Association. International Rules for Seed Testing. Seed Science and Technology (International Seed Testing Association, Bassersdorf, Switzerland), pp.5A-24.

International Seed Testing Association (ISTA). (2014b). Detection of *Xanthomonas campestris* pv. *campestris* on *Brassica* spp. Method description 7-019a, Annex to Chapter 7: Seed Health Testing Methods. www.seedtest.org/upload/cms/user/SH-07-019b-2014.pdf. Accessed 1/02/2013.

Kubota, M., Abiko, K., and Yanagisawa, Y. (2006). Frequency of *Alternaria brassicicola* in commercial cabbage seeds in Japan. J. Gen. Plant Pathol. *72*, 197-204 http://dx.doi.org/10.1007/s10327-006-0272-1.

Randhawa, P.S., and Schaad, N.W. (1984). Selective isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds. Phytopathology 74, 268-272.

Sangeetha, C.G., and Siddaramaiah, A.L. (2007). Epidemiological studies of white rust, downy mildew and Alternaria blight of Indian mustard (*Brassica juncea* (Linn.) Czern. and Coss.). Afr. J. Agric. Res. *2* (*7*), 305-308.

Sangvikar, V.R., and Wadje, S.S. (2012). *In vivo* testing of plant extracts against seed-borne pathogens. International Research Journal of Biological Sciences *1* (*6*), 1-4.

Sivapalan, A., and Browning, J.W. (1992). Incidence of *Alternaria brassicicola* (Schw.) Wiltsh. on *Brassica oleracea* seeds. Aust J Exp Agric *32*, 535-537 http://dx.doi.org/10.1071/EA9920535.

Tohyama, A., and Tsuda, M. (1995). Alternaria on cruciferous plants. *Alternaria* species on seed of some cruciferous crops and their pathogenicity. Mycoscience *36* (*3*), 257-261 http://dx.doi.org/10.1007/BF02268599

Uma, V., and Wesely, E.G. (2013). Seed borne fungi of rice from South Tamil Nadu. J. Acad. Indus. Res. *1*(*10*), 612-614.

Vermont Seed Standard Regulations. (2014). www.agriculture.vermont.gov/sites/ag/files/pdf/feed_seed_fert_lime/Seed/2008finalseedrule.pdf. Accessed 15/12/2015.

Wieser, A., Schneider, L., Jung, J., and Schubert, S. (2012). MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond (mini review). Appl. Microbiol. Biotechnol. *93* (*3*), 965-974 http://dx.doi.org/10.1007/s00253-011-3783-4.